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Patents ADP number *(if you know it)*
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METHODS

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METHODS

A new modulator of the Transforming Growth Factor-β (TGF-β) cell signalling pathway is described, namely an endogenous human protein designated BS69. Methods are provided to identify compounds that interfer with the biological activity of BS69 on the TGF-β cell signalling pathway.

TGF-β cell signalling failure is implicated in a number of different tumour types. Cell signal failure within other receptors in the TGF-β super family is implicated in other diseases such as arthritis, atherosclerosis, apoptosis, inflammation, wound healing and diabetic nephropathy. It is also known that administration of TGF-β helps prevent mucosistis and alopecia in patients undergoing chemotherapy or radiotherapy (PCT Publication No. 94/06459) and lowers resitance of multi-drug resitant malignant cells to chemotherapy (PCT Publication No. 92/13551).

TGF-β is one growth factor of a large super family which play broad roles in cell growth and differentiation in a variety of organisms. Examples of groups within the super family are the TGF-β set, activin/inhibin set, Mullerian inhibiting substance, glial cell line-derived neurotrophic factor and Bone Morphogenetic Proteins (BMPs).

Within the TGF-β subset the biological response to the growth factor is first initiated by binding of TGF-β to its respective receptor. The receptor is formed from two components TGF-β receptor I (TβR-I) and TGF-β receptor II (TβR-II) both of which contain cytoplasmic serine/threonine kinases and both of which are required for effective cell signalling (Wieser, R. et al., Mol. Cell. Biol. (1993) 13, 7239-7247).

It was not until recently that the biological molecules involved in signal cell transduction of TGF-β were discovered. Through screening of genes in transgenic Drosophila expressing only partially active decapentaplegic (DPP), which is equivalent in Drosophila to BMP in vertabrates, a new gene was found [Mothers against DPP(Mad)] which was able to restore the phenotype to the transgenic Drosophila expressing only partially active DPP protein (Sekelsky et al. Genetics (1995), 139, 1347-1359). Analysis of the Mad gene sequence showed it to be closely homologous to the sma genes of Caenorhalotibditis Elegans and the putative human tumour suppresor gene DPC4 (Deleted in Pancratic Carcinoma).

Such genes, of which several have now been identified, are now collectively referred to as Smad (Derynck, R. et al., Cell (1996), 87, 173).

The Smad proteins constitute a unique signalling pathway which convey signals directly from TGF-β type receptors to the nucleus, where they modulate gene transcription. There is close homology between many of the Smad proteins across species to a degree that Smad proteins from one species may elicit a response in a different species. Many Smad proteins (Smad 1,2,3 and others) are specific to the pathway associated with a particular receptor, others (Smad 4) act as a common mediator in different pathways. What is known at the current time for TGF-β signalling pathway is briefly described below, a full review can be found in Heldin, L., et al., Nature (1997), 390, 465.

Briefly described the mechanism of receptor cell signal transduction involves the following steps. In TGF-β signalling the TβRI and II receptors are activated by autophosphorylation following TGF-β binding to the receptor complex. Smad 2 and 3 associate with the activated receptor complex and are themselves phosphoylated at a characteristic C-terminal Ser-Ser-X-Ser motif. After activation of the Smad 2 and 3 heterodimer the common mediator Smad 4 forms a complex with the heterodimer, which in turn translocates to the nucleus where it directly or indirectly modulates gene transcription.

As described above the TGF-β signalling pathway is implicated in a number of different diseases and as such this biological mechanism represents an attractive target for intervention in treating such diseases.

BS69 (Hateboer, R. et al., EMBO Journal (1995), 14, 13, 3159-3169 and PCT Publication No. WO 97/00323) is described as being an adenovirus EI-A-associated protein which inhibits EIA adenovirus gene transactivation. A later disclosure (Kurozumi, K. et al., (1998), 3, 4, 257-264) describes an alternatively spliced, and considerably shorter, form of BS69, which they call BRAM1 (BMP Receptor Associated Molecule 1), as being able to complex intracellularly with the BMP receptor.

We have found through the use of protein hybridisation studies that the protein BS69 complexes with the Smad 2 and 3 proteins.

Whilst BMP is a member of the TGF-β superfamily of growth factors/receptor types it is known that BMP does not elicit signal transduction through Smad 2 or 3 proteins. In addition Kuroumi et al. found that BS69 did not complex with the BMP receptor and,

therefore, no cellular function for the BS69 protein was described. We have discovered a new cellular mechanism of action for the BS69 protein.

We present as the first feature of the invention a method for the discovery of a modulator of BS69 activity, which method comprises contacting an assay system capable of presenting information on the effects of a chemotherapeutic agent on the activity of BS69 or a derviative thereof with a potential chemotherapeutic agent under conditions in which BS69 is active in the absence of the potential chemotherapeutic agent and measuring the extent to which the potential chemotherapeutic agent is able to modulate the activity of BS69.

Preferably BS69 activity may be described as the binding of BS69 to a human BS69 binding substrate. A "human BS69 binding substrate" is a protein endogenously expressed in human cells which is capable of having its biological function modulated by binding of BS69. Preferably the human BS69 binding substrate is selected from Smad 2, Smad 3, a complex of Smad 2 and Smad 3, and a complex of Smad 2, Smad 3 and Smad 4 (herein after called BS69 binding substrate), more preferably the BS69 binding substrate is Smad 2 or Smad 3.

Potential chemotherapeutic agents which may be tested in the screen include those molecules, whether simple organic molecules, for example, of less than 2000 Daltons or larger biologic molecules, such as peptides, antibodies or DNA/RNA sequences, which may modulate the biology or pharmacology of BS69 activity, for instance by affecting the protein:protein binding of BS69 to a human BS69 binding substrate or by modulating the expression of DNA or RNA which encodes BS69. Suitable molecules include simple organic molecules, mimetics, nucleotide sequences, antibodies and any other molecules that modulate the activity of BS69.

It will be appreciated that there are many assay systems which may be employed to perform the present invention. Examples of assay sytems used to detect chemotherapeutic agents which may modulate the biological or pharmacological activity of BS69 are:

In vitro proximity assays such as a scintillation proximity assay (SPA), as described in Udenfield et al., Anal. Biochem. (1987), 161, 494. In SPA derivatised microspheres which contain a scintillant and a fluorophore are used which attach through the derivatised group to a biological molecule of interest. When the biological molecule of interest binds a radiolabelled molecule then the proximity of the radiolabel to the scintillant causes increased emmission of radiation signal and measurable increases in flourophore excitation. In the present case, for

example, BS69 is bound to a scintillant/fluorophore containing microsphere through, for example, a streptavidin/biotin bridge, and a human BS69 binding substrate is radiolabelled or bound to a support with a radiolabel. Any potential chemotherapeutic agent which affects the way in which BS69 binds to the human BS69 binding substrate will affect the radiation emitted by the system. Alternatively instead of radiolabels and scintillants flurophore donor and acceptor molecules may be used in what is called homogeneous time resolved flourescence (HTRF), for example the acceptor fluorophore is XL 665 and the donor fluorophore is europium (CIS Bio.). A further preferred feature of the invention is the invention as defined above wherein the assay system is a proximity assay, preferably SPA or HTRF.

In vitro cellular assay systems may be used. For example a measurable output of BS69 activity could be detected when a reporter gene is placed under the control of the TGF-β signal transduction pathway. A heterologous cell is created which has a reporter gene under transcriptional control of the TGF-β pathway and which also expresses BS69, such a cellular assay may be prepared as described in US 5,436, 128. Genes under TGF-β control which may be replaced by a reporter gene, for example by homozygous recombination, include plasminogen activator inhibitor-1, $p15^{ink4b}$, and p^{WAF1}, Attisano et al., Biochemica et Biophysica Acta 1222, 71-80, Hannon and Beach, Nature 371, 257-261 and Datto et al., J.Biol.Chem., 270, 28623-28628. In this way, stimulation or inhibition of signal transduction results in stimulation or inhibition of reporter gene activity and potential chemotherapeutic agents which interfer with BS69 activity may be detected. Suitable reporter genes include the β-galactosidase lac Z gene of E.Coli (Casadaban et al., Meth. Enzymol. (1983), 100, 293-308) or the Firefly luciferase gene (de Wet et al., Proc. Nat. Acad. Sci. USA (1985), 82, 7870-7873).

We present as a feature of the invention a method for the discovery of a modulator of BS69 activity, which method comprises contacting a potential chemotherapeutic agent with a heterologous cell comprising a reporter gene, expression of the reporter gene being under the control of the TGF-β signal pathway which is in turn under the control of BS69, a promoter which is activated by the TGF-β signal pathway and which has the gene encoding the reporter protein under its control, and determining modulation of BS69 by the potential chemotherapeutic agent by reference to any change in the expression of the reporter gene.

Preferably the measurement of reporter gene expression is compared with a control cell construct wherein the reporter gene is under the control of the TGF-β signal pathway but in which BS69 is not expressed.

The promoter may be a naturally occurring promoter for TGF-β signalling, or it may be a synthetic promoter responsive to the TGF-β transduction pathway. Synthetic promoters would comprise one or more response elements to the signalling pathway, as well as elements such as a TATA box, required for correct transcription initiation.

The components of the TGF-β signalling pathway may be endogenously expressed within the cells used in such asssay, for example by the use of mammalian cell lines. Alternatively, components, such as heterologous receptors, may be expressed so that they couple to the TGF-β signalling pathway. Also, an endogenous component may be removed, for example by gene deletion, and replaced with an exogenous protein which will restore the function of the pathway.

An alternative *in vitro* cellular system is the two-hybrid assay system. The two-hybrid system uses the ability of a pair of interacting proteins to bring a transcription activation domain into close proximity with a DNA-binding site that regulates the expression of a reporter gene. Commercially available systems such as the CLONTECH, Matchmaker™ systems and protocols may be used with the present invention. (*See also*, Mendelsohn, A.R., Brent, R., Curr. Op. Biotech., 5:482 (1994); Phizicky. E.M., Fields, S., Microbiological Rev., 59(1):94 (1995); Yang, M., et al., Nucleic Acids Res., 23(7):1152 (1995); Fields, S., Sternglanz, R., TIG, 10(8):286 (1994); US Patents 5,283,173 and 5,468,614). Two hybrid screening systems can be practiced either with a positive readout or with a negative readout. Recently, some examples of "reverse" two-hybrid systems have been described. Leanna, C.A. and Hannick, M. (1996) Nuc. Acids Res. 17 3341-3347 use an output in which a gene under the control of the two hybrid system is toxic in the presence of cycloheximide. Vidal, M., Brachmann, R., K., Fattaey, A., Harlow, E. and Boeke, J.D. (1996) Proc. Natl Acad. Sci. U.S.A. 93, 10315-10320 use the property of the URA3 gene product that it can be selected against by 5-fluoro-orotic acid. It is possible to test the ability of a potential chemotherapeutis agent to interfer between the binding of BS69 and a human BS69 binding substrate where BS69 is expressed as a fusion protein to a part of a transcription factor, either the transcription activation domain or the DNA-binding site, and the human BS69 binding substrate is

expressed as a fusion protein to the other part of the transcription factor. Wherein if hybridisation of the transcription factor is prevented from occuring by a chemotherapeutic agent then transcription of a reporter gene under transcriptional control of the transcription factor is interupted.

Several variations on the two hybrid system are known, and may be configured for use in the present invention. For example, a "tribrid" system has been described in which the two hybrid interaction will only occur if one component is phosphorylated by a kinase introduced into the cell (Osborne, M.A., Dalton, S. and Kochan, J.P. (1995) Bio/Technology 13, 1474-1478).

We present as a feature of the invention a method for the discovery of a modulator of BS69 activity, which method comprises contacting a potential chemotherapeutic agent with a heterologous cell comprising a transcription factor dependant promoter, a reporter gene under the control of the transcription factor dependant promoter, a fusion protein of BS69, or a human BS69 binding substrate, and a domain of a transcription factor which binds to the promoter and a second fusion protein of a human BS69 binding substrate, or BS69, and a domain of the transcription factor which activates transcription, wherein binding of BS69 to the human BS69 substrate causes the two domains of the transcription factor to become disposed to promote expression of the the reporter gene, and determining modulation of BS69 activity by the potential chemotherapeutic agent by reference to any change in the expression of the reporter gene.

As described above an alternative approach to the intervention of BS69 binding to a human BS69 binding substrate is to affect gene transcription or gene translation in the cell and thus prevent BS69 protein production in the cell. A variety of points in these processes may be disrupted such as by interference by a chemotherapeutic agent in the binding of BS69 transcription factors to the upstream promoter sites or by a chemotherapeutic agent binding to the coding DNA or mRNA (such as anti-sense nucleotides) of BS69.

Assay methods which may be utilised in the performance of the above aspect of the invention include those disclosed in European Publication No. 0483249.

Compounds that modulate the expression of DNA or RNA encoding the BS69 polypeptide may be detected by a variety of assays. The assay may be a simple "yes/no" assay to determine whether there is a change in expression of a reporter gene. The assay may

be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample. Systems in which transcription factors are used to stimulate a positive output, such as transcription of a reporter gene, are generally referred to as "one-hybrid systems" (Wang, M.M. and Reed, R.R. (1993) Nature 364, 121-126). Using a transcription factor to stimulate a negative output (growth inhibition) may thus be referred to as a "reverse one-hybrid system" (Vidal et al, 1996). Therefore, in the present invention a reporter gene is placed under the control of a BS69 promoter.

Therefore in a futher aspect of the invention we provide a heterologous cell wherein expression in the cell of a reporter gene is under the control of a BS69 transcription factor dependent promoter, and wherein expression of the transcription factor is inducible, whereby inhibition of gene transcription by the potential chemotherapeutic agent may be determined by reference to a lack of expression of the reporter gene.

We also provide a method for identifying inhibitors of BS69 transcription which method comprises contacting a potential therapeutic agent with a heterologous cell as described above and determining inhibition of BS69 transcription by the potential therapeutic agent by reference to a lack of expression of the reporter gene under transcriptional control of the BS69 transcription factor dependant promoter.

In general, eukaryotic transcription factors consist of a DNA binding domain and a transcription activation domain (Ptashne (1988) Nature 335, 683-689). Frequently these factors are dimers. Thus there may be three interfaces at which interference with a chemotherapeutic agnet may inhibit a transcription factor: the DNA:protein interface, the dimerisation interface, and the interface between the activation domain and the transcription apparatus (Peterson, M.G. and Baichwal, V.J. (1993) TibTech 11, 11-18). To find inhibitors of the interaction of mammalian DNA binding protein with its binding site, a transcription activation domain is fused to that DNA binding domain in order to make a transcription factor which functions in the cell type of interest. Conversely, if the interaction between an activation domain and the transcription machinery is of interest, a DNA binding domain may be fused to the activation domain of interest to yield a transcription factor. In such circumstances, it may also be desirable to express within the cell the protein which the activation domain contacts. Generally activation domains are believed to activate transcription through recruitment of the RNA polymerase holoenzyme (Ptashne, M. and

Gann, A. (1997) Nature 386, 569-577). This recruitment occurs through protein:protein interactions. Using genetic techniques it is possible to substitute components of the *S. cerevisiae* holoenzyme for mammalian homologues. In this way the protein:protein interaction of interest may be reconstituted using components from the same species.

Reference to BS69 its polypeptide, DNA or RNA sequences include references to derivatives thereof. Derivative polypeptides, or DNA/RNA sequences, of BS69 include

i) allelic variations of BS69, in particular any single nucleotide polymorphism SNP;
ii) a fragment of BS69, i) or iii) capable of binding to BS69 binding substrate; and
iii) a mutant form of BS69, i) or ii),

and, preferably, exclude BRAM1.

Particularly preferred polypeptide fragments are those which are at least 15 amino acid long and include wholly or at least partially the Smad binding domain of BS69.

For the purposes of this application the gene DNA and amino acid sequence of BS69 referred to herein are disclosed in PCT Publication No. WO 97/00323 SEQ ID NO:1 and 2.

Allelic variations or SNPs in the BS69 DNA sequence may be detected by alteration in the pattern of restriction fragment length polymorphisms capable of hybridising to SEQ ID NO:1 of WO 97/00323 or by the inability of allele-specific oligononnucleotide probes to hybridise to SEQ ID NO:1 of WO 97/00323.

It will be readily appreciated by the skilled reader that as a result of the degeneracy of the genetic code a multitude of sequences, may having minimal homology to any naturally occuring gene for BS69, may be produced and found to have utility in the present invention. Thus, the invention contemplates each and every possible variation of nucleotide sequence based on possible codon choices coding for the same amino acid.

Monospecific antibodies to BS69 may be purified from mammalian antisera containing antibodies reactive against the polypeptide or are prepared as monoclonal antibodies reactive with the BS69 using the technique of Kohler and Milstein, Nature, 256:495 (1975). Mono-specific antibody as used herein is defined as a single antibody

species or multiple antibody species with homogenous binding characteristics for BS69. Homogenous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope BS69 specific antibodies are raised by immunizing animals such as mice, rats, guinea pigs, rabbits, goats, horses and the like, with rabbits being preferred, with an appropriate concentration of BS69 either with or without an immune adjuvant and optionally conjugated toa carrier protein such as albumin.

Further features of the invention include:

A method of treatment of a patient in need of such treatment for a condition which is mediated by the biological or pharmacological activity of BS69 on a BS69 human binding substrate, comprising administration of a polypeptide substantially as depicted in WO97/00323 SEQ ID NO:2 or a pharmacologically active fragment thereof.

A method of treatment of a patient in need of such treatment for a condition which is mediated by the biological activity of BS69 on a BS69 human binding substrate, comprising administration of a nucleic acid substantially as depicted in WO97/00323 SEQ ID NO:1 or the anti-sense sequence or a biologically-effective fragment of either thereof.

A method of treatment of a patient in need of such treatment for a condition which is mediated by the biological activity of BS69 on a BS69 human binding substrate, comprising administration of an anti-sense molecule.

A compound that modulates the biological or pharmacological activity of BS69 on a BS69 human binding substrate identified by the method of the invention as described above.

A pharmaceutical composition comprising a compound that modulates the biological or pharmacological activity of BS69 on a BS69 human binding substrate identified by the method of the invention as described above.

A method of treatment of a patient in need of such treatment for a condition which is mediated by the pharmacological or biological activity of BS69 on a BS69 human binding substrate comprising administration of a modulating compound identified by the method of the invention as described above.

Examples

In order to identify novel modulators of the TGF-beta signalling pathway we employed the two hybrid screening methodology. The two hybrid system can be used in order to detect expressed proteins from a cDNA library that interact with a protein of interest. The protein that we used as bait was Smad2. Smad2 is one of the pathway specific Smads and is known to lie on the TGF-beta pathway from receptor to nucleus. Smad2 has been shown to be in a complex with Smad4 and Fast-1 on the TGF-beta inducible Xenopus promoter, Mix.2 .

Two Hybrid Screen Construction

Human full length Smad2 was isolated by PCR from a human brain cDNA library (OriGene Technologies, Rockville MD.) and cloned into PCRScript® (Stratagene). The oligonucleotide primers used for the PCR synthesis were:

5'GTCCCGGGATGTCGTCCATCTTGCCATTCACG3'
5'GTGTCGACTTATGACATGCTTGAGCAACGCAC3'

The full length human Smad2 insert was excised from the PCRScript® using Sma1 and Sal1 restriction endonucleases and cloned into the two hybrid bait vector pGBD-1 (James, Genetics 144: 1425-1436) resulting in a N-terminal Gal4 DNA binding domain fusion with full length human Smad2 (Gal4::Smad2). In order to verify that full length Smad2 was interaction competent, Xenopus Fast-1 and human Smad4 were cloned into the activation domain fusion vector PGAD-1 (AD::Fast1, AD::Smad4). It had been previously shown that these proteins will interact with human Smad2 (Chen, Nature 398(4), 85-88). As such, the Smad2/Fast-1 and Smad2/Smad4 interaction was used as a positive control for the ability of Smad2 to interact with other proteins in the yeast two hybrid system.

Human full length Smad4 was isolated in a similar manner to Smad2 from a human skeletal muscle cDNA library. The primers used for PCR were :

5'GTCCCGGGATGGACAATATGTCTATTACGAAT3'
5'GTGTCGACTCAGTCTAAAGGTTGTGGGTCTGC3'

Xenopus Fast-1 was inserted into PCRScript® by amplification of a cloned Xenopus Fast-1 sequence (C.Hill, ICRF, London) and then excised with Sma1 and Bgl11 restriction endonuclease and cloned into pGAD-1. The primers used for PCR were:

5'GTCCCGGGATGAGAGACCCCTCCAGTCTGTAC3'
5'GTAGATCTCTATTTCATTGAGTGTCCACTCCA3'

As a negative control human Smad1 was isolated and cloned into PGAD-1 (AD::Smad1). Smad1 does not interact with Smad2 *in vivo* or *in vitro*(Zhang et al. Nature 383,168-172, Lagna, G., et al Nature 383, 832-836). The primers used for the PCR reaction were:

5'GTCCCGGGATGAATGTGACAAGTTTATTTTCC3'
5'GTGTCGACTTAAGATACAGATGAAATAGGATT3'

The yeast two hybrid system used is that of Vidal et al., PNAS USA 93, 10321. The S. Cerevisiae screening strain, MAV203, has three reporter genes (HIS3, URA3, and LacZ) stably integrated in single copy numbers at different loci in the yeast genome. Interaction of an activation domain fused protein with a DNA bound protein of interest will result in induction of the His3, Ura3, and LacZ reporter genes allowing growth of MAV203 on medium lacking histidine and uracil, and producing blue colonies when assayed with X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside). MAV203 was transformed with combinations of the different control plasmids and were scored on their ability to grow on medium lacking uracil and histidine, and producing blue color. This was done on a scale of +++, meaning very high, to -, meaning none.

Table 1 indicates that when GAL4::Smad2 is transformed into MAV203 on its own it cannot activate any of the reporter genes. If Smad2 is co-transformed with AD::Fast-1 a strong interaction is observed between the two as indicated by very high activation of the

reporter genes. A similar strong interaction is observed between the Smad2 and AD::Smad4 fusion proteins in the two hybrid assay. Contrasting this, no interaction was observed between Smad2 and AD::Smad1 and none of the activation domain tagged proteins were able to activate the reporter genes on their own. This indicates that the GAL4::Smad2 fusion protein is functional in its ability to interact with other proteins.

A screen was then performed in order to identify proteins that interact with Smad2. MAV203 was co-transformed with GAL4::Smad2 and a human skeletal muscle two hybrid cDNA library (Clonetech). 5.9 x 10^6 independent co-transformants were assayed for their ability to interact with Smad2. Five different proteins were isolated and showed varying ability to interact with GAL4::Smad2 from very strong (+++, 1 isolate), strong (++, 2 isolates) and weak (+, 2 isolates) as assayed by their ability to activate all three reporter genes. The cDNA containing plasmids were isolated and co-transformed with GAL4::Smad2 as a check for "true positive". All five retained that ability to activate the three reporter genes.

The strongest interactor, Cl.51, was isolated independently in the screen 33 times. Sequence analysis of the longest isolate of Cl.51 indicated that it was a previously identified protein BS69. BS69 was identified as a protein that interacts with the adenoviral 289R E1A gene product and in doing so inhibits its ability to activate transcription (Hateboer, R. et al., EMBO 14(13):3159-3169). BS69 is a 562 amino acid protein and has a single corresponding mRNA species of approximately 4.7 kb. The longest isolate of BS69 in the present two hybrid screen was 2200bp, encoding the C terminal region of the protein from amino acid XX to 562 and the 3' untranslated region. This area of the C terminus of BS69 is the E1A interaction domain (Hateboer, R. et al., EMBO 14(13):3159-3169). It is therefore evident that the area of BS69 that mediates interaction with E1A also mediates interaction with Smad2.

In order to assess the specificity of the BS69/Smad2 interaction, the other members of the Smad family, as well as a totally unrelated protein, peroxisome proliferating antigen receptor gamma (PPAR-G), were examined for protein-protein interaction with BS69 in the two hybrid system. Each of the proteins were cloned from PCR reactions in a manner similar to that for SMAD2 into the pGBD-1 vector in order to make GAL4 DNA binding domain fusions (see table 2 for specific primer sequence and library used for template). Table 3 summarises the results from the BS69 specificity two hybrid analysis. BS69 interacts very strongly with Smad2 and Smad3. A very weak (+/-) interaction is obtained with Smad1

which may not be physiologically relevant. No interaction is observed with Smad4, Smad5, Smad6, or Smad7. BS69 does not interact with PPAR-G and cannot activate the reporter genes on its own. This indicates that the BS69 interaction observed is specific to Smad2 and Smad3 (possibly Smad1) suggesting a role for BS69 in modulating the activity of TGF-beta through Smad2 and/or Smad3. As BS69 was identified as a protein that interacts with E1A, inhibiting its ability to activate transcription, the same may be true in relation to the TGF-beta pathway. Smad2 and Smad3 have both been shown to contain transcription activation function. They heterodimerise with SMAD4, translocate to the nucleus, and activate TGF-beta responsive genes. BS69 may function cellularly as an inhibitor of TGF-beta induced transcriptional activation by interacting with Smad2/Smad3 and inhibiting their ability to activate transcription.

Table 1: GAL4::Smad2 Interaction Verification

Co-Transformant	Strength
-	-
AD::Fast-1	+++
AD::Smad4	+++
AD::Smad1	-

MAV203 were transformed with GAL4::Smad2 and the indicated co-transformant. They were then assayed for strength of interaction on their ability to activate the URA3, HIS3, and LacZ reporter gene.

Table 2: cDNA PCR Primers

Protein	Primer Sequence
Smad3	5'GTCCCGGGATGTCGTCCATCCTGCCTTTCACT3'
	5'GTGTCGACCTAAGACACACTGGAACAGCGGAT3'
Smad5	3'GTCCCGGGATGACGTCAATGGCCAGCTTGTTT3'
	5'GTGTCGACTTATGAAACAGAAGATATGGGGTT3'
Smad6	5'GTCCCGGGATGTCCAGAATGGGCAAACCCATA3'
	5'GTGTCGACCTATCTGGGGTTGTTGAGGAGGAT3'
Smad7	5'GTGAATTCATGTTCAGGACCAAACGATCTGCG3'
	5'GTGTCGACCTACCGGCTGTTGAAGATGACCTC3'

Table 3: BS69 Specificity Interaction

Co-Transformant	Strength
GAL4::Smad1	+/-
GAL4::Smad2	+++
GAL4::Smad3	+++
GAL4::Smad4	-
GAL4::Smad5	-
GAL4::Smad6	-
GAL4::Smad7	-
GAL4::PPAR-G	-

MAV203 were transformed with AD::BS69 and the indicated co-transformant and assayed for ability to interact. Strength of interaction is assayed by ability to activate URA3, HIS3, and LacZ reporter gene.

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